Genotyping of Infectious bronchitis viruses isolated from broiler chicken farms in Iran during 2015-2 016

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Abstract:

BACKGROUND: Avian infectious bronchitis is considered as an important viral disease worldwide. Genotyping based on the S1 subunit of spike protein gene of the causative agent, avian infectious bronchitis virus, can be used to classify IBV isolates. **OBJECTIVES:** This survey was carried out to characterize the infectious bronchitis virus (IBV) genotypes circulating in Iran and determine their prevalence rate. **METHODS:** In this survey, 100 samples of trachea were collected from broiler chickens suspected of IBV during 2015 to 2016. Nested polymerase chain reaction (Nested PCR) followed by S1 gene sequencing was applied to genotype the detected isolates. **RESULTS:** In this survey, forty-five isolates were detected and classified in four distinct genotypes, variant 2 [IS/1494/06], 4/91, QX and Massachusetts, based on phylogenetic analysis. The prevalence rates of the variant 2 [IS/1494/06], 4/91, QX and Massachusetts were 66.67%, 24.45%, 4.44% and 4.44%, respectively. **CONCLUSIONS:** This survey demonstrates the epidemiology of IBV genotypes in Iran and provides an insight into the evolution of these strains. Moreover, it is clarified that IBV genotypes prevalence is constantly changing in a region.

Introduction

Infectious bronchitis (IB) is a viral respiratory disease that can cause major economic losses to the poultry industry all over the world. In addition to respiratory system, IB can affect other organs such as kidney and ovary (Cavanagh, 2007). The causative agent of the disease, infectious bronchitis virus (IBV), is classified as a member of coronaviridae family, genus Gammacoronavirus. This enveloped virus possesses a positive sense, single stranded RNA with a size of 27 kb. IBV genome encodes four structural proteins: spike, membrane, small membrane, and nucleocapsid (Montassier, 2010). The spike glycoprotein consists of 2 subunits: S1 and S2. The S1 subunit plays the main role in cell attachment, induction of serotype specific and neutralizing antibodies (Feng et al., 2014). It is known that most variations occurring in the IBV are related to the S1 sequence, as it contains three hypervariable regions mostly prone to

mutations and recombination (Najafi et al., 2016). So far, a large number of IBV serotypes have been detected (Cavanagh, 2007). Methods such as hemagglutination inhibition test, virus isolation, virus neutralization, ELISA and PCR can be used to detect different IBV serotypes in a geographical area (Villarreal, 2010). Recently molecular methods based on S1 gene sequencing has been widely used due to its efficiency in characterizing IBV genotypes and variants (Seger et al., 2016).

As other parts of the world, outbreaks of IB frequently occur in Iran. Since the first molecular identification of IBV serotypes by Seify abad Shapouri et al. (2002), various serotypes and variants have been identified in Iran. Recently Hosseini et al. (2016), Najafi et al. (2016) and Homayounimehr et al. (2016) have investigated circulating IBV genotypes in Iran.

The aim of the current survey was to characterize circulating IBV variants isolated from broiler chicken farms of 8 provinces of Iran by using sequencing method in conjunction with nested polymerase chain reaction (Nested-PCR) during 2015-2016.

Materials and Methods

Sampling and preparation: In order to conduct the survey, 100 samples of trachea were collected from broiler chickens showing signs resembling infectious bronchitis during 2015 and 2016. For monitoring the distribution of virus genotypes, sampling was done in eight provinces of Iran. The provinces included East Azerbaijan, Qazvin, Isfahan, Khorasan, Khuzestan, Kordestan, Mazandaran and Semnan. Samples from each bird were homogenized, and a 10 % (w/v) suspension was made in PBS. Subsequently, samples were vortexed for 20 min and then centrifuged at 2000 rpm for 5 min (Najafi et al., 2016; Seger et al., 2016).

RNA extraction and cDNA synthesis: To extract the RNA, the CinnaPure RNA Extraction Kit was used as described by the manufacturer. The procedure of reverse transcription to yield cDNA involved 2 steps. At first, 5 µl of RNA and 1 µl of random hexamer were mixed and incubated at 65 oC for 5 min. After that, 7.25 µl DEPC-treated water (SinaClon, Iran), 2 µl dNTP mix (SinaClon, Iran), 0.25 µl RiboLock RNase Inhibitor (Thermo Fisher Scientific, USA), 0.5 µl Revert Aid Reverse Transcriptase (Thermo Fisher Scientific, USA), and 4 µl 5X RT Reaction Buffer were added to each tube with final volume of 20 µl. The process of incubation consists of 25 °C for 5 min, 42 °C for 60 min, 95 °C for 5 min, and 4 °C for 1 min (Seger et al., 2016).

Nested PCR: To conduct the genotyping process, the method of nested PCR was chosen. First round PCR was performed by mixing a 20 µl volume in a mixture containing 2 µl of sterile distilled water, 2 µl of the SX1 (50-CACCTAGAGGTTTGYTWGCATG-30) and SX2 (50-CACCTCTATAAACACCYTTAC-30) primers, 3 µl cDNA, and 13 µl Sinaclon 2X PCR master mix (Sinaclon, Iran). The process was done in an Eppendorf Master Cycler gradient thermocycler (Eppendorf, Hamburg, Germany) and the steps were as follows: denaturation period at 94 ºC for 2 min and 35 cycles of denaturation for 15s, annealing at 58 ºC for 30s, and polymerization at 72 ºC for 30s and final step was extension at 72 ºC for 10 min. For nested PCR, a 2.5µl aliquot of a 1:50 dilution of the first amplicon was put for the next step by using the primers SX3 (50-TAATACTGGY AATTTTTCAGATGG-30)

Table 1. Prevalence rate of identified IBV genotypes isolated from each of eight provinces of Iran during 2015-2016.

	Total	Negative	Positive	Variant 2	793/B	Mass	QX	IR-1	IR-2	Positive percent
East Azerbayjan	16	9	7	4	3	0	0	0	0	43.75
Qazvin	13	5	8	5	2	0	1	0	0	61.54
Isfahan	11	6	5	4	0	1	0	0	0	45.45
Khorasan	13	8	5	2	2	0	1	0	0	38.46
Khozestan	11	6	5	3	2	0	0	0	0	45.45
Kordestan	13	7	6	3	2	1	0	0	0	46.15
Mazandaran	13	9	4	4	0	0	0	0	0	30.77
Semnan	10	5	5	5	0	0	0	0	0	50
Total	100	55	45	30	11	2	2	0	0	45

Table 2. Percent identity of partial nucleotide sequences of the S1 glycoprotein genes of some Iranian IBVs to those of IBV reference strains.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1 UT-GKHG7		99.57	79.95	81.58	79.97	82.14	99.57	98.7	99.57	97.36	79.42	82.14	82.14	81.67	82.22
2 UT-GKHG9			79.95	81.58	79.97	82.14	99.13	98.7	99.13	96.91	79.42	82.14	82.14	81.67	82.22
3 UT-GKHG14				94.22	78.25	97.38	79.37	81.58	80.52	80.52	71.02	97.38	97.3	80	79.42
4 UT-GKHG24					78.81	96.04	81.01	83.17	82.14	82.14	71.42	96.04	96.04	80.55	79.97
5 UT-GKHG 26						80.5	79.4	80	79.97	80.55	72.35	80.5	80.5	98.7	98.26
6 UT-GKHG 33							81.58	83.72	82.7	82.7	72.19	100	100	82.19	81.63
7 Iraq/IS-1494/SGK-34								98.27	99.13	96.91	78.84	81.58	81.58	81.11	81.67
8 Iran/IS1494/UT-IVO93									98.27	96.03	79.44	83.72	83.72	81.68	81.68
9 IS/1494/06										97.81	79.42	82.70	82.70	81.67	82.22
10 Variant2 (AF093796)											78.82	82.70	82.70	82.22	82.77
11 H120												72.19	72.19	74.22	74.22
12 4/91 vaccine													100	82.19	81.63
13 Iraq/793-b/SGK-1														82.19	81.63
14 Iran/QX/PCRLab															99.57
15 Iran/QX/UT-IVO 105															

and SX4 (50-AATACAGATTGCTTACAACCACC-30) and similar cycling program to first round PCR.

The final products were subjected to electrophoresis with 1.5% agarose gels in Tris/Borate/EDTA (TBE) buffer, then stained with GelRed™ (Biotium, USA) and visualized under UV light (Seger et al., 2016).

Phylogenetic analysis: The AccuPrep® PCR Purification Kit (Bioneer Co., Korea) was applied to purify the PCR products. Sequencing was performed with the primers

(both directions) used in the PCR. Chromatograms were evaluated with CromasPro (CromasPro Version 1.5). All sequences from a given sample were combined and used to NCBI-BLAST for confirmation and construct alignments. Sequence homology analysis was performed and Phylogenetic tree was constructed using MEGA7.0 with the neighbor-joining algorithm (bootstrap values of 1000) with the Kimura2 parameter model (Tammara et al., 2011). The obtained sequences were submitted to NCBI GenBank database with the accession numbers KX702149-KX702181

Results

Among 100 samples obtained from vaccinated broiler flocks showing respiratory problems in eight provinces of Iran, forty-five IBV isolates were identified. These field isolates and reference strains were phylogenetically compared by partial S1 nucleotide sequences analysis and results revealed that the isolates were classified in four distinct genetic groups (Table 1 and Fig. 1).

Group I included 30 field isolates (66.67 %) and showed maximum nucleotide sequence similarity to variant2 (IS/1494/06 strain). Group II comprised 11 field viruses (24.45 %) which were grouped with the 793/B serotype. In group III there were 2 isolates (4.44%) of the field isolates clustered with QX strain. Group IV contained 2 field isolates (4.44 %) that showed a close relationship to the Massachusetts strain.

As shown in Table 1, the highest prevalence of IBV occurred in Qazvin province; 62.5 % of the detected isolates were correlated with variant 2, 25 % were similar to the 793/B type and 12.5 % were grouped with the QX genotype. In contrast, Mazandaran province had the lowest prevalence rate of IBV. Among four IBV genotypes found in this survey, variant 2 was the only serotype found in all eight provinces whereas the QX strain was only detected in Qazvin and Khorasan provinces. It is noteworthy to consider that in two provinces (Mazandaran and Semnan) only variant 2 genotype was isolated. In addition, the results showed that IR-1 and IR-2 genotypes were not detected in any samples tested in this survey.

The isolates classified as variant 2 viruses in this survey showed more than 99.9 % similarity to IS/1494/06. Besides, their similarity to variant 2 (AF 093796) was 99.97%. Relevancy of variant 2 viruses with Iran/IS-1494/UT-IVO93 was higher than 98% while the rate of difference with Iraq/IS-1494/SGK-34 was around 0.004%.

In the 793/B-like viruses group, percent of variability with Iraq/793-B/SGK-1 was 0.04. In comparison with 4/91 (vaccine strain), these viruses showed 0.04% difference.

As it was concluded from the phylogenetic tree, one of the QX viruses isolated in this survey, UT-GKHG26, was 98.49% similar to Iran/QX/UT-IVO 105 and 97% to QX (AF193423). Moreover, this strain was 98.1% identical to Iraq/QX/SGK21 (KU143898). The other QX virus isolated, UT-GKHG27, was identical to Iran/QX/UT-IVO 105 and 98.26% like UT-GKHG26.

In the group of viruses found to be similar to Mass type viruses, UT-GKHG15 was identical to the reference strain H120 (JN600610) and M41 (KF188436).

Discussion

Infectious bronchitis is recognized as one

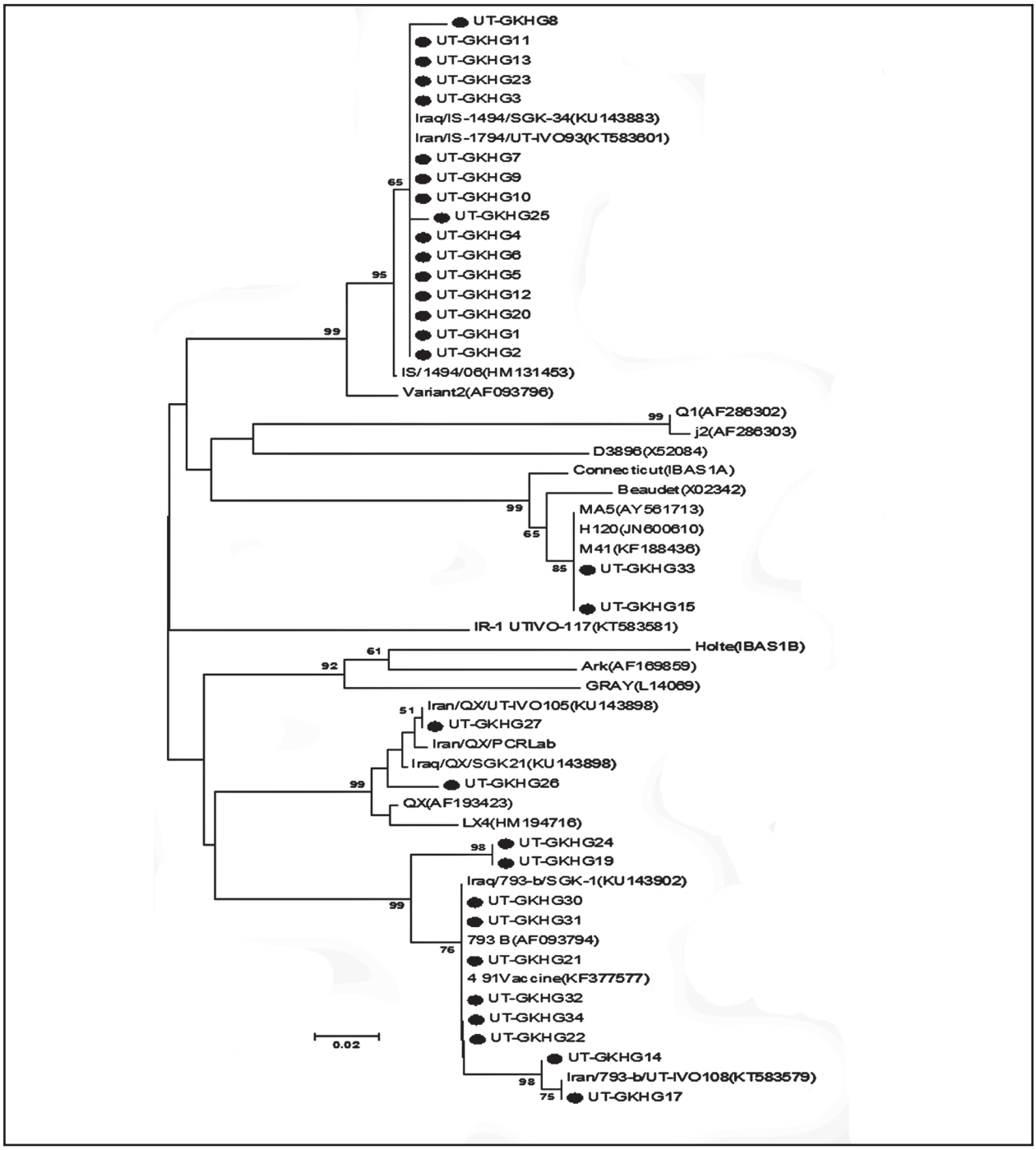

Figure 1. Phylogenetic tree of IBV isolates based on a partial sequence of the S1 gene.

of the most important poultry diseases all over the world. The virus has a highly infectious nature with various serotypes which may co-circulate in a region (Cavanagh, 2007). overally, cross protection among distinct types of the virus does not develop. Nevertheless, there are some strains of the virus that can be effective in generating cross protection against other serotypes and are defined as protectotypes (De Wit, 2000).

Since the first report of variant 2 isolate by Callison et al. (2001), this strain has been reported variably in other countries such as Turkey (Yilmaz et al., 2016), Libya (Awad et al., 2014), Iraq (Seger et al., 2016) and Egypt (Selim et al., 2013).

A survey carried out on genotyping Iraqi isolates by Seger et al. (2016) revealed that 46.87 % of detected strains belonged to variant 2 which was the most common isolate found in this work.

Abdel-ELGhany et al., (2015) conducted a survey of IB outbreaks in breeders and layers during 3 years from 2012 -2014 in Egypt. Their method was to amplify 500-bp of S1 gene targeting the HVR 3. They found 2 groups of isolates circulating in the farms. The first group was similar to variant 2 and D274 vaccine and the second group was like H120 and MA5 vaccine.

Yilmaz et al. in 2016 conducted a phylogeny survey of IBV on the broiler and layer flocks in Turkey. Their phylogenetic tree based on partial S1 sequences of the isolates showed that viruses detected in five broiler flocks were similar to the variant 2 strains.

In Iran, the results of Hosseini et al. (2015) survey by partial S1 sequencing showed that variant 2 virus was the second most common isolate found during the period 2010 to 2014.

Moreover, Homayounimehr et al. (2016) found 3 isolates related to variant 2 from 10 IBV strains detected by testing trachea, cecal tonsil, and kidney tissues collected from broiler and layer farms in Iran.

Najafi et al. (2016) detected 118 IBV isolates from tissue samples of broiler chicken farms, confirmed them by real-time PCR and characterized them by sequencing the S1 gene. They reported variant 2 isolate as the most prevalent strain (34%) in Iran during 2014- 2015 which is in complete agreement with our results.

Based on the results of our survey, prevalence rate of variant 2 was 67 percent of detected isolates that had a significant increase in comparison with the previous studies carried out in Iran. It has been proven that variant 2 is a nephropathogenic strain (Najafi et al. 2016). Furthermore, this serotype effect on respiratory tract, digestive system and renal tissue due to its epithe-liotropic nature is considered as an important factor in its resistance and survival in poultry farms in spite of different measures taken for prevention and control of the disease.

It has been shown that some vaccines combinations can protect chickens against variant 2 infection. Due to the lack of uniform prevention and control schedules and application of various vaccination methods in different regions of Iran, it is expected that prevention of IBV variants spread cannot be met completely. Moreover, important parameters in vaccine application like inadequate dose of the vaccine, lighting and ventilation can adversely affect disease control (De Wit et al., 2010).

Gelb et al. (2005) noted in a challenge study that the H120 vaccine provides poor protection, from 25% to 58% against variant 2. Therefore in a poultry practice relying solely on H120 vaccination, insufficient immunization is expected.

In a study conducted by Awad et al. (2016), 2 vaccinal strains including Massachusetts and CR88 (a 793B strain) were used in order to protect broiler chickens from two variant 2 isolates, IS/885/00-like and IS/1494/06-like. Their work revealed that application of live H120 and CR88 vaccines at day-old followed by CR88 vaccine at 14 days-old results in 100 percent protection from clinical signs and tracheal or kidney lesions. In other vaccination method, H120 at day-old followed by CR88 at 14 days-old, the tracheal ciliary protection from IS/1494/06-like infection was 80 percent.

The second genotype detected in this survey was 4/91 with the prevalence rate of 24.45%. This serotype was discovered in Iran in 1998 (Vasfi Marandi, et al., 1998) and so far several researchers have reported this serotype circulating in Iranian farms (Seyfi Abad Shapouri et al., 2002; Akbari et al., 2004; Ghahremani et al., 2011). Afterwards, vaccination was adopted to protect chickens against this strain. This study, in line with previous papers published lately, demonstrates the presence of 4/91 in most parts of Iran (Homayounimehr et al., 2016; Hosseini et al., 2015; Najafi et al., 2016).

One of the IBV genotypes recently distributed widely in Europe and Asia is QX strain, which was at first manifested with clinical signs such as proventriculitis and then mainly by nephritis and false layer syndrome. In Iraq, results of Seger et al.'s (2016) survey showed that 9.37% of detected isolates belonged to QX-like genotype which were like Chinese QX (AF193423) strain with 98.39 % homology. They also found 99.68 % similarity with the Iranian QX IBV strain (PCR Lab/06/2012).

In the present survey, we detected QX genotype in 4.44% of the isolates. In the previous studies done during 2010 to 2014 and 2014 to 2015 in Iran, the rate of this genotype was found to be 9.6% and 10 % of the detected field isolates, respectively (Hosseini et al., 2015; Najafi et al, 2016). It can be argued that by the passage of time the prevalence rate of this genotype faces a decreasing trend in Iran. The reasons leading to this event can be attributed to the application of different vaccines which have cross protection effect against QX genotype and also transferring maternal antibody from breeders to the offspring. Supporting this idea is the study conducted by Teregino et al. (2008) in which a combination of Ma5 and 4/91 vaccines applied at 1 and 14 days respectively inhibits infection and disease in chickens challenged with QX like variant. Moreover, Mohammadi et al. (2015) and Awad et al. (2015) noticed that concurrent vaccination with Mass and 793/B results in good protection against QX variants challenge.

Massachusetts serotype was the first type of IB viruses identified in Iran by Aghakhan et al. (1994), which causes disease outbreaks all over the world. Since then, vaccination based on Mass serotype and its derivatives have been administered in Iran.

Overall, this survey demonstrates the constant changing epidemiology of IBV and emphasizes the crucial role of vaccination in prevention and control of the disease. It can be concluded that consistent monitoring of IBV helps in better understanding of its evolution and the necessary measures for decreasing the prevalence rate of infection.

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چکیده

زمینه مطالعه: بیماری برونشیت عفونی طیور به عنوان یک بیماری مهم ویروسی در سراسر جهان در نظر گرفته می‌شود. ژنوتایپینگ براساس تحت واحد S۱ ژن پروتئین Spike عامل بیماری، ویروس بیماری برونشیت عفونی، می‌تواند در طبقه‌بندی جدایه‌ها مورد استفاده قرار بگیرد. **هدف:** این بررسی جهت شناسایی ژنوتیپ‌های در حال چرخش ویروس برونشیت عفونی و تعیین میان شیوع آن انجام گرفت. **روش کار:** در این بررسی ۱۰۰ نمونه نای از جوجه‌های گوشتی مشکوک به ویروس برونشیت عفونی در سال ۹۴-۹۵ جمع‌آوری گردید. روش Nested PCR و متعاقب آن سکانس ژن S۱ جهت تعیین ژنوتیپ جدایه‌های شناسایی شده اعمال شد. **نتایج:** در این بررسی، ۴۵ جدایه شناسایی گردید که بر اساس تجزیه و تحلیل فیلوژنتیکی در چهار ژنوتیپ مجزا دسته‌بندی گردید (واریانت ۲ [۰۶/۱۴۹۴/IS]، ۴/۹۱، QX و ماساچوست). میزان شیوع واریانت ۲، ۴/۹۱، QX و ماساچوست به ترتیب ٪۶۶/۶۷، ٪۲۴/۴۵، ٪۴/۴۴ و ٪۴/۴۴بود. **نتیجه‌گیری نهایی:** این بررسی اپیدمیولوژی ژنوتیپ‌های ویروس برونشیت عفونی و دیدی از تکامل این ویروس را نشان می‌دهد. همچنین روشن گردید که شیوع ژنوتیپ‌های ویروس برونشیت عفونی در یک منطقه دائماً در حال تغییر می‌باشد.

واژه های کلیدی: تعیین ژنوتیپ، ویروس برونشیت عفونی، واکنش زنجیره‌ای پلیمراز آشیانه‌ای، شیوع، توالی یابی

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